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Anticancer activities of anthocyanin extract from genotyped *Solanum tuberosum* L. "Vitelotte"

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ABSTRACT

The action of anthocyanins contained in the Vitelotte potato (*Solanum tuberosum* L.) in both breast and haematological cancers were investigated. The biomedical activities of anthocyanin extract derived from the Vitelotte cultivar were determined. Molecular genotyping was performed to properly identify this outstanding genotype in comparison to other potato varieties and to promote the utilization of this genetic resource by plant breeders. Furthermore, cellular and molecular characterization of the action of anthocyanin extract in cancer cells revealed that modulation of cell cycle regulators occurs upon treatment. As well as inducing apoptotic players such as TRAIL in cancer systems, anthocyanin extract inhibited Akt-mTOR signalling thereby inducing maturation of acute myeloid leukaemia cells. These results are of interest in view of the impact on food consumption and as functional food components on potential cancer treatment and prevention.

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1. Introduction

Epidemiological analyses suggest that individuals consuming a diet rich in fruits and vegetables have a reduced risk of developing non-communicable diseases such as cancer. The beneficial effects of a plant-based diet have been attributed to the presence of bioactive compounds, including phenols, which act as free radical scavengers. Among cultivated plants, the potato (*Solanum tuberosum* L.) exceeds all other crops in terms of calories, proteins, and several other nutrients (Niederhauser, 1993). It is also a very good source of fibre, potassium, and vitamin C. Depending on its genetic background, the potato also represents one of the most copious sources of phytochemicals, such as polyphenols including anthocyanins, essential for human diet. There are over 5000 potato varieties worldwide, with very diverse tuber characteristics, including a broad range of colours, shapes, sizes, and tastes. According to tuber flesh colour, varieties can be categorized into two main groups: white/yellow tubers, and tubers with intensely coloured (blue, purple, or red) flesh. Pigmented potatoes have attracted particular attention not only for their appealing colour and excellent taste, but also because of their high anthocyanin content. The pigments have in fact been identified as anthocyanins (Eichhorn & Winterhalter, 2005). In nature, anthocyanins are known to improve defence mechanisms and, consequently, plant fitness. Similar to anthocyanins from other plants, it has been suggested that analogous potato anthocyanins may also be important functional food ingredients (Brown, 2005). Due to their well-documented beneficial effects on human health, anthocyanins are of major scientific interest. These bioactive compounds play a key role in defence against oxidative cell damage and in the prevention of degenerative diseases, including ageing, cancer, diabetes, and cardiovascular diseases (Bontempo et al., 2013). Potatoes are the fourth-largest food crop feeding the global population, and we regularly consume greater quantities of the potato tuber compared to fruits and vegetables, above all in developing countries. Pigmented potatoes may therefore represent one of the world's most important anticancer foods, and are also gluten-free.

Besides standard and internationally known potato cultivars, some local heritage varieties have specific quality attributes, such as high dry matter content and low reducing sugar content. They also possess noteworthy historical and culinary characteristics. The Vitelotte variety has been used in French cuisine since the early 19th century and is characterized by natural colouring with dark blue, almost black, skin and violet flesh. The medium-sized elongated tubers have a sweet taste with a nutty flavour and chestnut scent (Hillebrand et al., 2010; Hillebrand, Naumann, Kitzinski, Kohler, & Winterhalter, 2009). The tuber colour is due to the abundant presence of anthocyanin pigments, a subclass of flavonoids. Interestingly, the anthocyanin composition of the tubers is very uncommon, containing petunidin and malvidin derivatives as major pigments (Bontempo et al., 2013; Hillebrand et al., 2009, 2010; Ieri, Innocenti, Andrenelli, Vecchio, & Mulinacci, 2011).

The aim of the study has been to verify the effects of crude and anthocyanin extract derived from Vitelotte and to improve our knowledge on the potential beneficial effects of this potato arising from its functional components. To reach these goals the cellular and molecular mechanisms of crude and AE-induced cancer cell differentiation were determined. In addition, guarantying the specificity of the potato variety, molecular genotyping was also carried out, properly identifying the genetic profile of Vitelotte.

2. Materials and methods

2.1. Plant materials

For both simple sequence repeats (SSR) analysis and biological assays, plant materials (leaves and tubers, respectively) of Vitelotte were collected at Nusco (Avellino) in Southern Italy. Tubers were washed in running tap water, cut into slices of 0.5 mm in width, dried in a heated air dryer, and then pulverized by the disintegrator. After drying, the weight of potatoes was 157 g. Samples were kept at 4 °C. DNA for SSR profiling comparisons was extracted from the leaves of 12 additional varieties: Agata, Agria, Asterix, Badia, Bartina, Inova, Marabel, Primura, Sieglinde, Spunta, Vivaldi, and Volumia. Plants were grown under greenhouse conditions at the Department of Agricultural Sciences, University of Naples Federico II.

2.2. Chemicals

Methanol was purchased from Carlo Erba (Milan, Italy). Oil Red O and Hematoxylin solution, Harris modified were purchased from Sigma Aldrich (St. Louis, MO, USA), Isopropanol was purchased from Merck-Millipore (Billerica, MA, USA), Everolimus was purchased from BioVision (Milpitas, CA, USA).

2.3. Preparation of anthocyanin extract (AE)

For the preparation and characterization of the crude extract (CE) see Gellatly et al. (Gellatly, Moorhead, Duff, Lefebvre, & Plaxton, 1994) and for anthocyanin extract (AE) see Bontempo et al. and references therein (Bontempo et al., 2013). Briefly, pigmented potato powder was put into a 50 mL conical flask, then added in acid–ethanol (HCl, 1.5 mol/L) with a solid–liquid ratio 1:32 (w/v) and put in thermostatic water bath at a selected temperature (80 °C) for 60 min, then, centrifuged at 3220 *g* for 15 min. The supernatant was collected and transferred into a 50 mL volumetric flask for the determination of anthocyanin yield. About 1 g of the samples was used for each treatment.

2.4. DNA genotyping

Potato genomic DNA was isolated in duplicate from a pool of fully developed young leaves from three different plants of each genotype using the protocol previously reported (De Masi et al., 2007). DNA integrity and quality were checked by gel electrophoresis and spectrophotometric analysis. Molecular analyses of SSR loci were carried out by using eight nuclear microsatellite primer pairs (Supporting Information Table S1), as previously described (Ghislain et al., 2009). These SSR loci were recommended by the International Potato Center

(http://www.cipotato.org) based on quality criteria, genome coverage, and locus-specific information. In brief, SSR amplifications were performed via touchdown polymerase chain reaction (PCR) in a 20 μL volume containing 1× GoTaq Reaction Buffer (Colorless, USA) with 1.5 mM $MgCl_2$, 100 μM of each deoxynucleoside triphosphate (dNTP), 5 pmol of both the fluorescently labelled forward and reverse SSR primers, 1 unit of GoTaq DNA polymerase (Promega, Madison, WI, USA) and 40 ng of potato genomic DNA. The forward primers were labelled with fluorophore 6-carboxyfluorescein (6-FAM) or hexachloro-6-carboxyfluorescein (HEX). Touchdown PCR was carried out on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) in two separate phases. First cycling was as follows: initial DNA denaturation for 4 min at 94 °C, DNA amplification for 5 cycles of 45 s at 94 °C, 1 min at 5 °C above standard annealing temperature (T*a*), decreasing by 1 °C per cycle, and 30 s at 72 °C. Subsequently, DNA amplification continued for 30 cycles of 45 s at 94 °C, 1 min at T*a*, and 30 s at 72 °C, with a final extension step of 20 min at 72 °C. Amplicons were brought to optimal dilution (40–100×) after gel electrophoresis visualization. Then, 1 μL was added to a final volume of 10 μL in HiDi formamide (Applied Biosystems, Foster City, CA, USA) and 0.3 μL of GeneScan 500 ROX Size Standard (Applied Biosystems), as internal molecular weight standard for size calibration of PCR products. The prepared amplicons were denatured at 95 °C for 5 min and then separated by capillary electrophoresis run on an ABI PRISM 3130 DNA Analyzer (Applied Biosystems). Detection of SSR alleles was performed using the 3130 Data Collection software v3.0 (Applied Biosystems). Two biological replicates were analysed for each locus. SSR alleles of each genotype were scored by GeneScan Analysis software (Applied Biosystems) as present (1) or absent (0). The information content of microsatellite loci was estimated both by polymorphic information content (PIC), according to the formula $PIC = 1 - \Sigma p_i^2$, where p_i is the frequency of the ith allele detected in all individuals of the population (Nei, 1973), and by power of discrimination (PD), according to the formula given above, but with allele frequency replaced by genotype frequency (Kloosterman, Budowle, & Riley, 1993). A similarity matrix was calculated with the Dice coefficient (Sneath & Sokal, 1973) using the program DendroUPGMA (http://genomes.urv.es/UPGMA/) (Garcia-Vallve, Palau, & Romeu, 1999). Using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm, it was possible to construct a tree diagram (dendrogram) to illustrate the genetic clustering of the potato varieties under investigation.

2.5. *Cell lines, culture conditions and cell vitality*

U937, MDA-MB-231 (human) and 3T3-L1 (mouse) cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). U937 cells were grown at 37 °C in 5% CO_2 atmosphere in RPMI-1640 medium (Gibco, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 1% ampicillin/streptomycin and 0.1% gentamicin. 3T3-L1 fibroblasts were grown in formulated Dulbecco's Modified Eagle's Medium (DMEM, Gibco), completed with bovine calf serum to a final concentration of 10%. To evaluate cell vitality cells were diluted 1:1 (v/v) in Trypan blue (Sigma) and counted. Experiments were performed in triplicate.

2.6. *Differentiation assay*

Granulocytic and monocytic differentiation was carried out as previously described (Altucci et al., 2001; Nebbioso et al., 2005). Briefly, U937 cells were harvested and resuspended in 10 μL phycoerythrin-conjugated CD11c (CD11c-PE) or 10 μL fluorescein isothiocyanate (FITC)-conjugated CD14 (CD14-FITC) (Pharmingen, San Diego, CA, USA). Control samples were incubated with 10 μL PE or FITC-conjugated mouse IgG1, incubated for 30 min at 4 °C in the dark, washed in PBS and resuspended in 500 μL PBS containing 0.25 μg/mL propidium iodide (PI). Samples were analysed by fluorescence-activated cell sorting (FACS) with Cell Quest technology (Becton Dickinson, San Diego, CA, USA). PI-positive cells were excluded from the analysis.

For adipogenic differentiation, treated 3T3-L1 cells were washed twice with PBS and fixed with 10% formalin. After fixation, cells were stained with filtered Oil Red O solution (stock solution: 3 mg/mL Oil Red O in isopropanol; working solution: 60% Oil Red O stock solution and 40% distilled water) for 1 h at room temperature. Cells were then washed with water, differentiated with a 60% isopropanol solution for 1 min, rinsed in water, stained in Mayer's hematoxylin, visualized by light microscopy, and photographed.

2.7. *Cell cycle analysis and evaluation of pre-G1 phase*

For cell cycle analysis and evaluation of pre-G1 phase, samples were processed as previously reported. Briefly, samples were resuspended in PBS-1× containing PI (50 μg/mL), sodium citrate (0.1%) and NP40 (0.1%), and then analysed using the FACScalibur flow cytometer with ModFit technology (Becton Dickinson). Apoptosis was measured as pre-G1 DNA fragmentation as previously reported (De Bellis et al., 2014; Lepore et al., 2013).

2.8. *Western blot analyses*

Forty micrograms of total protein extracts were separated on a 12% polyacrylamide gel and blotted as previously described (Nebbioso et al., 2011). Western blots were run for p21 (dilution 1:500; Becton Dickinson), p16, cyclin A, D1, D2, Rb (dilution 1:500; Santa Cruz, Santa Cruz, CA, USA) and p53 (dilution 1:500; Upstate, Billerica, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, dilution 1:500; Santa Cruz) was used to normalize for equal loading.

To quantify TNF-related apoptosis inducing ligand (TRAIL), 100 μg total protein extract was separated on 10% polyacrylamide gel and blotted as previously reported (Altucci et al., 2001, 2005). Western blots were performed using TRAIL (dilution 1:200; Abcam, Cambridge, UK); GAPDH (dilution 1:500, Santa Cruz) was used for equal loading. To determine ERK2, pERK, Akt, p70S6K, p-p70S6K, p-p90RSK and c-Myc expression levels, 35 μg of total protein extracts was separated on a 12% polyacrylamide gel and blotted. Antibodies used were: ERK2 (Santa Cruz, dilution 1:500), pERK (Santa Cruz, dilution 1:200), Akt (Cell Signaling, Danvers, MA, USA, dilution 1:1000), p70S6K and p-p70S6K (Merck-Millipore, dilution 1:1000), p-p90RSK (Cell Signaling, dilution 1:1000) and c-Myc (Santa Cruz, dilution 1:500). Total ERKs were used to normalize for equal loading. 5 μg of histone

extracts were separated on a 15% polyacrylamide gel and blotted as previously described (Nebbioso et al., 2011). Western blots were run for H3K9-14ac (Diagenode, Liege, Belgium). Total histone H4 levels were used to normalize for equal loading.

3. Results

3.1. *SSR markers characterize* S. tuberosum *L. var. Vitelotte*

DNA genotyping was carried out to identify and characterize the exact genotype profile of Vitelotte. This approach was considered relevant to specifically connect the biological effects to genotypes. All markers could be considered as they successfully amplified scorable alleles. Polymorphic SSRs were distributed on eight (out of 12) potato chromosomes. A total of 42 alleles in eight SSR loci were detected (Supporting Information Table S1), with an average of 5.25 alleles per locus. Only the two alleles 172 bp (locus STM1053) and 298 bp (locus STM5114) were present in all varieties, while the other 40 showed varying degrees of polymorphism (Table 1). The number of alleles per marker varied from three (loci STM1052 and STM1053) to nine (locus STG0001) (Supporting Information Table S1). For the eight SSRs, the average PIC was 0.69, varying from 0.36 per STM1053 to 0.83 per STG0001. PD values were very similar and varied between 0.90 and 0.92. SSR analysis allowed the identification of five private (i.e., genotype-specific) alleles in unpigmented cultivars: 154 bp at locus STM1106 in Volumia; 187 bp and 190 bp at locus STI0012 in Marabel and Spunta, respectively; 250 bp and 253 bp at locus STM5127 in Inova and Sieglinde, respectively (Table 1). Two private alleles were found in Vitelotte: 145 bp at locus STG0001 and 175 bp at locus STM1053 (Table 1). The UPGMA dendrogram derived from SSR analysis is presented in Fig. 1. It allowed us to graphically assign individuals to groups based upon the degree of similarity of genetic data. The genetic distances among potatoes studied here varied from 0.17 (between Agria and Badia) to 0.59 (between Bartina and Sieglinde), with an average value of 0.39 (data not shown). The dendrogram shows that Vitelotte clusters apart, displaying an independent status.

Our findings demonstrate that SSR analysis is useful for reliable identification of Vitelotte and provides a clear picture of its genetic background in comparison to the other varieties studied.

3.2. *Anthocyanin extract from* S. tuberosum *L. var. Vitelotte induces differentiation of cancer cells*

In a recent work we showed the content and quantification of anthocyanins derived from *Solanum tuberosum* L. var. Vitelotte. In addition, we found that the anthocyanin-rich extract displays anticancer action, activating molecular pathways of programmed cell death in both solid and haematological models of cancer (Bontempo et al., 2013). To investigate whether this action is accompanied by activation of maturation-related pathways, we tested the action of previously characterized AE versus

Table 1 – Alleles (bp) detected at 8 microsatellite loci in 12 potato varieties. Private alleles for each variety and locus are in bold.

Variety	STG0001	STI0012	STI0032	STM1052	STM1053	STM1106	STM5114	STM5127	Total alleles per genotype
Agata	129, 139, 143	165, 168, 171, 174	107, 119	210	169, 172	141	289, 292, 298	241, 244, 271	19
Agria	127, 139, 143	165, 168, 171, 184	107, 116, 119, 122	210, 219, 228	169, 172	157	286, 289, 298	241, 244	22
Asterix	127, 135, 139, 143	165, 168, 171	107, 119	210	172	137, 141	286, 298	241, 244, 271	18
Badia	127, 139, 143	168, 171, 174, 184	116, 119, 122	228	172	157	286, 289, 298	241, 244, 274	19
Bartina	120, 135, 139	171, 174	116, 122	210	172	157	289, 298	241, 244, 274	15
Inova	120, 127, 135, 143	165, 168, 171	107, 119	210	172	157	289, 292, 298	241, 244, **250**, 274	19
Marabel	125, 127, 139, 143	168, 184, **187**	107, 119, 122	219, 228	172	157	286, 292, 298	241, 244	19
Primura	129, 135, 143	168, 171, 174	119, 122	210	172	141	286, 289, 298	244, 271, 274	17
Sieglinde	127, 132, 143	168, 171, 184	107	210, 228	172	137, 141	286, 289, 298	241, **253**, 271, 274	19
Spunta	127, 135, 143	165, 184, **190**	110, 119, 122	210, 219, 228	169, 172	157	289, 298	241, 244, 274	20
Vitelotte	127, 132, 139, **145**	165, 168	107, 116, 122	210, 219	172, **175**	141	284, 289, 292, 298	241, 244	20
Vivaldi	139, 143	168, 171	110, 122	210, 228	169, 172	141, 157	284, 289, 292, 298	241, 244, 274	19
Volumia	120, 125, 139, 143	168, 171, 184	119, 122	219, 228	172	**154**	289, 298	241, 244, 271, 274	19

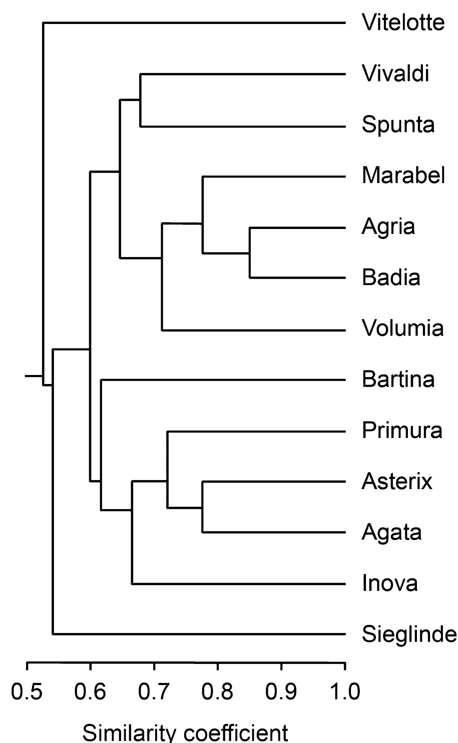

Fig. 1 – Tree diagram showing the genetic relationships between the 13 *Solanum tuberosum* varieties used in this study. The genetic distance between varieties was estimated using DNA polymorphism from eight SSR markers. The tree diagram was constructed through the UPGMA clustering method. The similarity on the x-axis is based on the Dice's coefficient.

CE on the regulation of CD11c and CD14 taken as markers of granulocyte and monocyte differentiation in acute myeloid leukaemia (AML) U937 cells (Fig. 2A). Anthocyanin-enriched extract increased the induction of differentiation pathways at the same concentration of the CE extract. Interestingly, AE was able to induce robust monocyte differentiation and weak granulocyte maturation in a dose-dependent manner, suggesting that the induction of differentiation might contribute to and strengthen AE-mediated anticancer action. In support of a more generalized differentiative activity induced by AE, 3T3-L1 cells, a model of potential adipocyte maturation, also responded to the treatment with evident intracellular fat droplets (Fig. 2B). In detail, the increase of fat droplets was quantifiable as 70 to 90% increase of positive cells upon the treatment with 2 to 5 mg/mL of AE. Note that the increase concerned both the number of cells and the amount of fat droplet staining per cell, suggesting that AE action on differentiation is a general feature of anthocyanins derived from Vitelotte.

3.3. *Anthocyanin extract from* S. tuberosum *L. var. Vitelotte induces cell cycle modulation*

To analyse at molecular level the differentiative effects detected in AML cells upon AE exposure we investigated the impact on cell cycle progression and checkpoints. Treatment induced p16, p21, p53 and Rb up-regulation, suggesting that pathways involved in cell cycle arrest are modulated. In agreement, cyclin D1, D2 and A were down-regulated, indicating a stop in cell cycle progression. Accordingly and as previously reported (Bontempo et al., 2013), apoptotic pathways involving TRAIL were induced (Fig. 3A).

3.4. *Anthocyanin extract from* S. tuberosum *L. var. Vitelotte inhibits Akt-mTOR signalling in haematological cancers*

Exposure of U937 cells to AE (2.5 and 5 mg/mL for 10 min, 25 min, 2.5 h and 24 h) showed constitutive expression protein levels of Akt (Fig. 3B). By contrast, both expression levels and phosphorylation of p70S6K were down-regulated, suggesting an inhibition of the mTOR complex. In agreement, phosphorylation levels of p90RSK were also repressed, corroborating an effect of AE in inhibiting mTOR and inducing differentiation. Supporting our hypothesis, c-Myc, a known target of mTOR, was repressed at the early time points. In contrast, when the effect of AE was tested in presence of Everolimus (Nishioka, Ikezoe, Yang, Koeffler, & Yokoyama, 2008), a known inhibitor of the mTOR pathway, no evident synergy in anticancer action was detected, with the pre-G1 peak induced by AE alone being comparable to that of Everolimus and AE in combination (Fig. 4).

Taken together these results indicate that AE inhibits Akt and mTOR signalling, but that its anticancer action is likely also due to a plethora of additional effects.

3.5. *Anthocyanin extract from* S. tuberosum *L. var. Vitelotte impacts on Akt-mTOR signalling in different models of cancer*

Exposure of MDA-MB-231 breast cancer cells to AE (5 mg/mL for 24 h) alone or in combination with SAHA, Everolimus or both showed a net decrease of the expression levels of p53 only in the combined case (Fig. 5). In agreement with the data shown in Fig. 3, p21 and cyclin A were up-regulated by AE. The fact that p21 up-regulation seems more stable in respect to the one detected in haematological models and that cyclin A is upregulated may reflect differences and specificities of the cell models. The down-regulation of cyclin D2 observed seems to be a general action. In full agreement, expression levels of p70S6K and AKT were down-regulated, suggesting that inhibition of the mTOR complex is a general feature exerted by AE also in solid cancer models such as the MDA-MB-231 breast cancer cells. Supporting our hypothesis, c-Myc, a known target of mTOR, was repressed at the early time points. Despite this, when the effect of AE was tested in presence of Everolimus again, no evident synergy in the anticancer action was detected. Note that corroborating and strengthening this hypothesis, when H3 acetylation levels where tested, only the effects related to the HDAC inhibition exerted by SAHA were clearly defined and no activity was detected in the combo treatment (Fig. 5).

Taken together these results indicate that AE impacts on mTOR signalling also in solid cancer models, but that its anticancer action is likely also due to a plethora of supplementary effects.

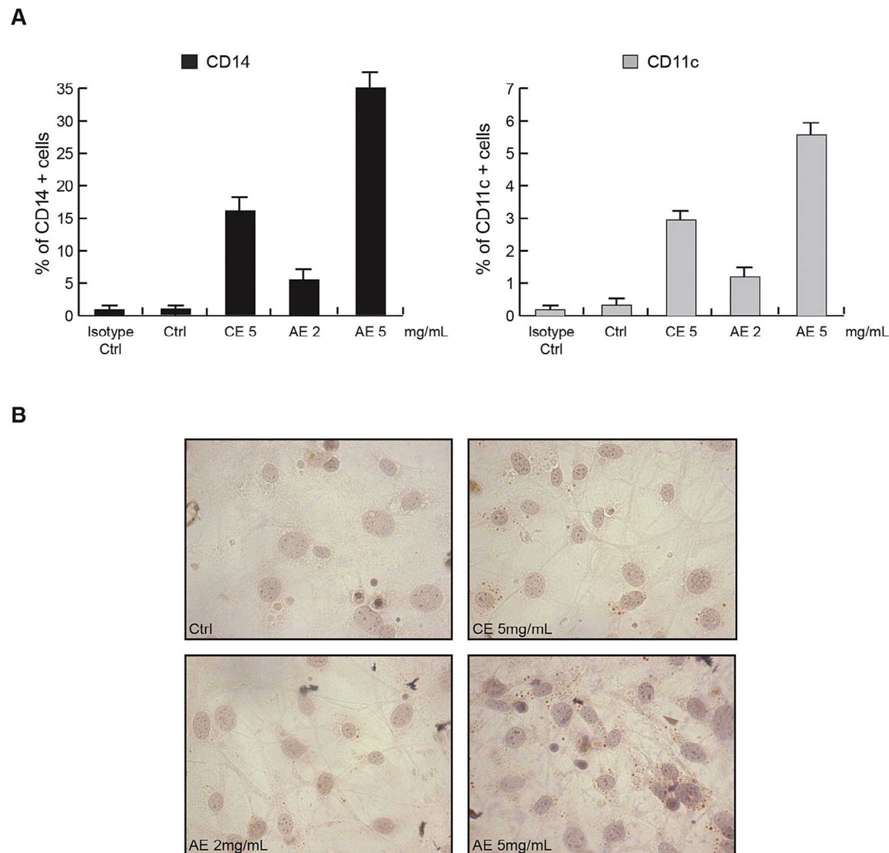

Fig. 2 – Anthocyanins induce cell differentiation. (A) FACS analysis of CD14 (left) and CD11c (right) expression in U937 cells upon crude extract (CE) and AE treatments at the indicated doses for 24 h. Error bars represent the standard deviation from two independent experiments carried out in duplicate. Isotype controls were used as negative control. (B) Oil Red O staining showing adipogenic differentiation in 3T3-L1 cells treated with TE and AE at the indicated doses.

4. Discussion

As one of the most versatile cultivated plants, the potato is cultivated worldwide for either food, or feed or industrial material production. It is also a plant with surprising medicinal properties recognized since its first introduction into Europe (Frusciante et al., 2000). Recent years have seen increasing interest in the search for new phytochemicals in potato, given that it produces a vast and diverse array of secondary metabolites, including the anthocyanins. Anthocyanin-rich foods are, among the others, blackcurrant juice, red grape juice, red wine, elderberries, blueberries and strawberries. Consumption of these foods has been associated with a reduced risk of human diseases and cancer, but the absorption and bioavailability of these molecules represent critical aspects for their potential role in disease prevention. The absorption, distribution, metabolism and elimination of the anthocyanins after oral administration have been recently summarized (Fang, 2014). The systemic bioavailability of anthocyanins is estimated to be 0.26–1.8%, lower than that of other flavonoids (Wu, Cao, & Prior, 2002), and their maximum plasma levels are of 1–100 nmol/L in the native form upon ingestion of doses of 0.7–10.9 mg/kg body weight.

In this work we have characterized the biomedical activities of both CE and AE derived from Vitelotte, an old and underutilized potato variety that has already demonstrated antioxidant and antimicrobial actions as well as anti-proliferative effects in cancer cells (Bontempo et al., 2013). We have also already reported the identification and quantification of anthocyanins contained in AE from Vitelotte (Bontempo et al., 2013). As first step, we performed a molecular study to determine the DNA fingerprint of Vitelotte in comparison to widely grown potato varieties. SSR markers were used for this purpose in that they are largely used in plants to detect differences in short tandem repeated DNA sequences (Kalia, Rai, Kalia, Singh, & Dhawan, 2011). Based on DNA polymorphisms, Vitelotte clustered apart with respect to the other potato varieties used here. In this variety we identified private alleles with interesting potential applications, as recently proposed also in *Catharanthus* (Lal, Mistry, Shah, Thaker, & Vaidya, 2011) and *Plumbago* (Haji et al., 2014). They could be useful not only for cultivar identification but also for marker-assisted selection when Vitelotte is used as a parental line in potato breeding programmes. Private alleles found in Vitelotte may have another important application if they are linked to its anthocyanin content, i.e., the possibility to track this important trait in segregating populations. In addition, private alleles may be used as

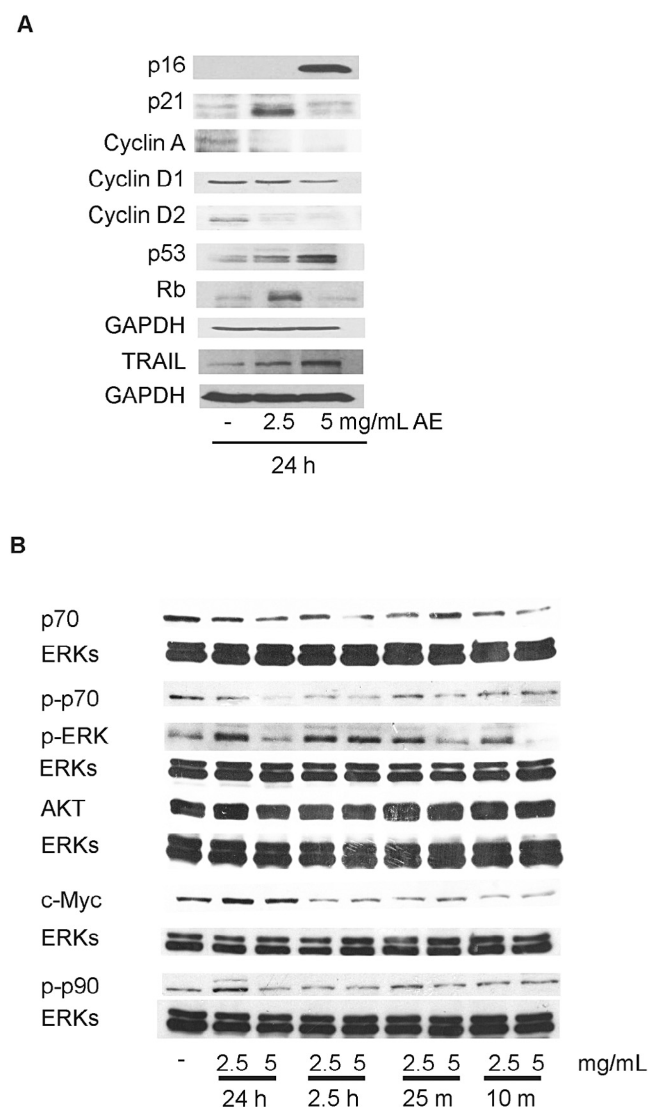

Fig. 3 – Anthocyanins modulate cell cycle phases and inhibit Akt-mTOR signalling. (A) Western blot analysis of expression levels of the indicated proteins in U937 cells after AE treatment at the indicated doses for 24 h. GAPDH was used as loading control. (B) Western blot analysis showing expression levels of the indicated proteins in U937 cells after AE treatment at the indicated doses and times. ERKs detection was used as loading control.

diagnostic markers to identify small distinct genomic regions within breeding materials with low polymorphism and redundancies among farmer-maintained clones of Vitelotte and to legally preserve it against frauds.

Following Vitelotte genetic profiling, the next step of our research was the characterization of the biomedical activities of crude extract versus AE derived from tubers of Vitelotte. Our results provided evidence that both CE and AE enhance differentiation pathways related to the specific inhibition of mTOR signal transduction. The fact that also the crude extract is able to induce differentiation (Fig. 2) is particularly relevant in view of the potential advantage for the consumption of this brand of potatoes. On the other hand, the enrichment of anthocyanins in the extract increases this activity, suggesting that food functionalization on this brand of potatoes might turn beneficial. These findings are in agreement with previous data showing the anticancer effects of anthocyanins *in vitro* as well as *in vivo* (Wang & Stoner, 2008). Anthocyanins are able to induce different responses, including radical scavenging activity, stimulation of phase II detoxifying enzymes, reduced cell proliferation, inflammation, angiogenesis and invasiveness. Additional effects comprise induction of apoptosis and differentiation, by activating genes involved in the PI3K/Akt, ERK, JNK, and MAPK pathways, and in their cancer chemo-preventive activity. *In vivo* studies have shown that dietary anthocyanins inhibit cancers of the gastrointestinal tract, and topically applied, these molecules inhibit skin cancer. Our data demonstrated that the induction of differentiation obtained using AE in both leukaemic and breast cancer systems is accompanied by c-Myc down-regulation, and modulation of p90 and ERK phosphorylation, suggesting that these signal transduction pathways might in part contribute to the establishment of the differentiated phenotype. Interestingly, a similar 'scenario' has been recently reported as mediated by the inhibition of Akt/mTOR pathways induced by histone deacetylase (HDAC) inhibitors in leukaemias (Nishioka et al., 2008). The fact that addition of the well-known mTOR inhibitor Everolimus (Nishioka et al., 2008) does not modify the induction of programmed cell death in our settings strongly suggests that pathways of maturation and cell death in AML cells might be distinct, or at least to some extent independent. This hypothesis is of particular interest in view of the potential importance of differentiation as well as cell death-inducing therapies in the treatment of cancer. Our finding is of specific significance given that mTOR modulators are currently in clinical trials against cancer (Motzer et al, 2008), and response to treatment might also be influenced by dietary factors. It is tempting to speculate that a combinatorial scheme of mTOR modulators together with different types of diets might at least partially improve treatment outcome. Despite our current growing knowledge on anthocyanin effects, we are still far from identifying the dose required to display at least part of them. Pharmacokinetic data indicate that the major absorption of anthocyanins is in the gastrointestinal tract (where they are metabolized by bacteria into a series of different and smaller bioavailable compounds) and is minimal in the blood. This evidence highlights the importance to further investigate the role of anthocyanin metabolites.

In the literature different works show conflicting results regarding the identity and prevalence of the major metabolites after the consumption of anthocyanin-rich foods (Cooke, Steward, Gescher, & Marczylo, 2005; Stoner et al., 2005; Wang & Stoner, 2008). *In vitro* studies concluded that bacterial metabolism involves the cleavage of glycosidic linkages and breakdown of anthocyanidin heterocycle forming phenolic acids such as protocatechuic, vanillic, syringic, caffeic and ferulic acids, aldehydes and their subsequent methyl, glucuronide and sulfate conjugation (Williamson & Clifford, 2010). It is plausible that the observed benefits of a rich-anthocyanin diet are due to the complex mixture of metabolites that remain in tissues and biological fluids for a longer time and higher dose than the parent anthocyanins. Besides, it is still unclear whether (i) anthocyanin concentrations *in vivo* are sufficient to elicit

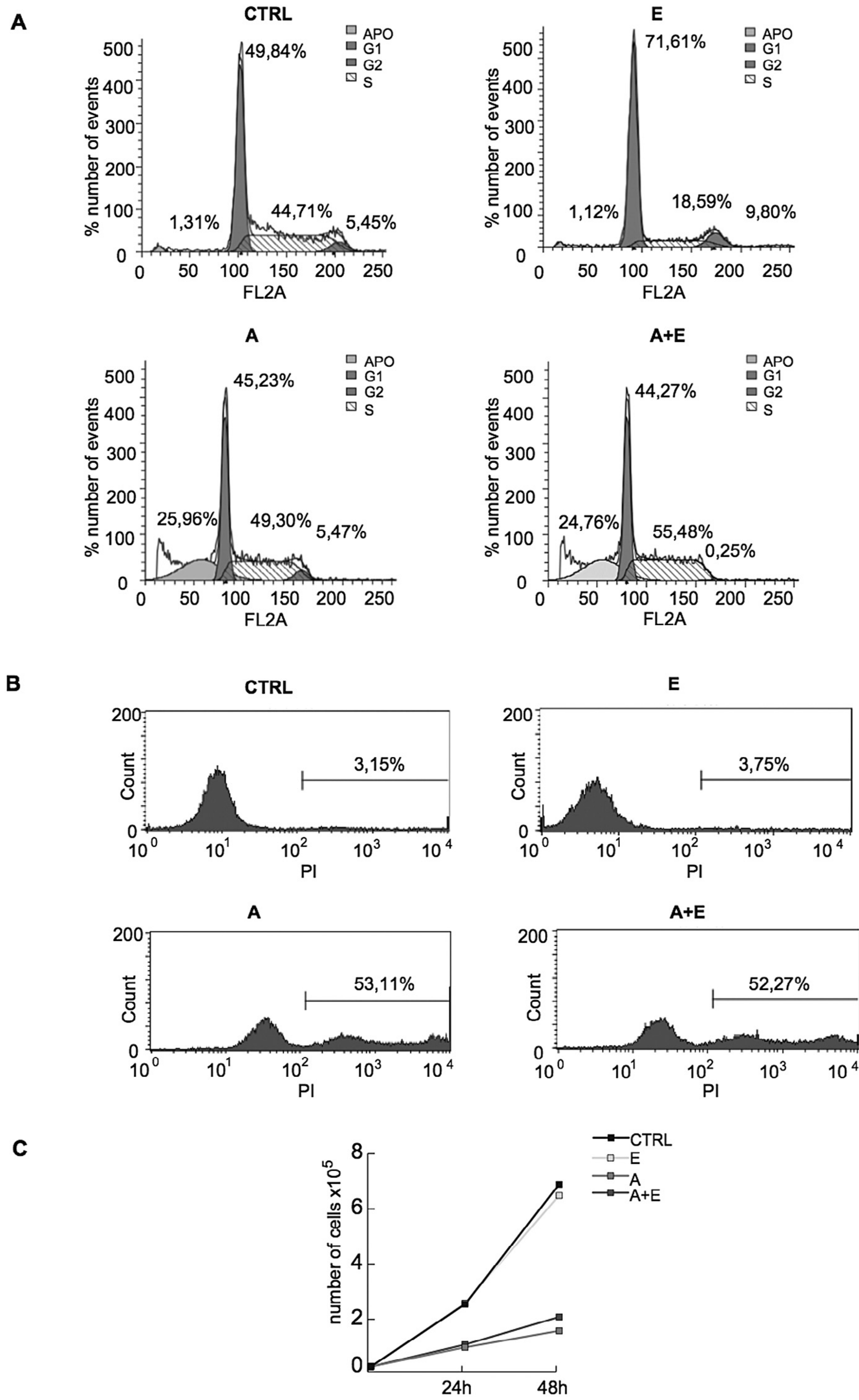

Fig. 4 – Effect of anthocyanins in presence of Everolimus by FACS analysis. (A, B) Representative DNA fluorescence histograms of two independent experiments carried out in duplicate of PI stained cells. U937 cells were untreated and treated with AE, Everolimus (Ev) and AE + Ev. (A) Cell cycle analyses at 24 h. The percentage of cells in pre-G1, G1, S and G2 phases were shown. (B) FACS analyses of apoptosis at 48 h with AE, Everolimus (Ev) and AE + Ev. The percentage of cell death in U937 cells was measured and shown. (C) U937 cell growth curves by trypan blue staining to measure viable cells treated as in A and B at 24 and 48 h. Error bars represent the standard deviation of two independent experiments carried out in duplicate.

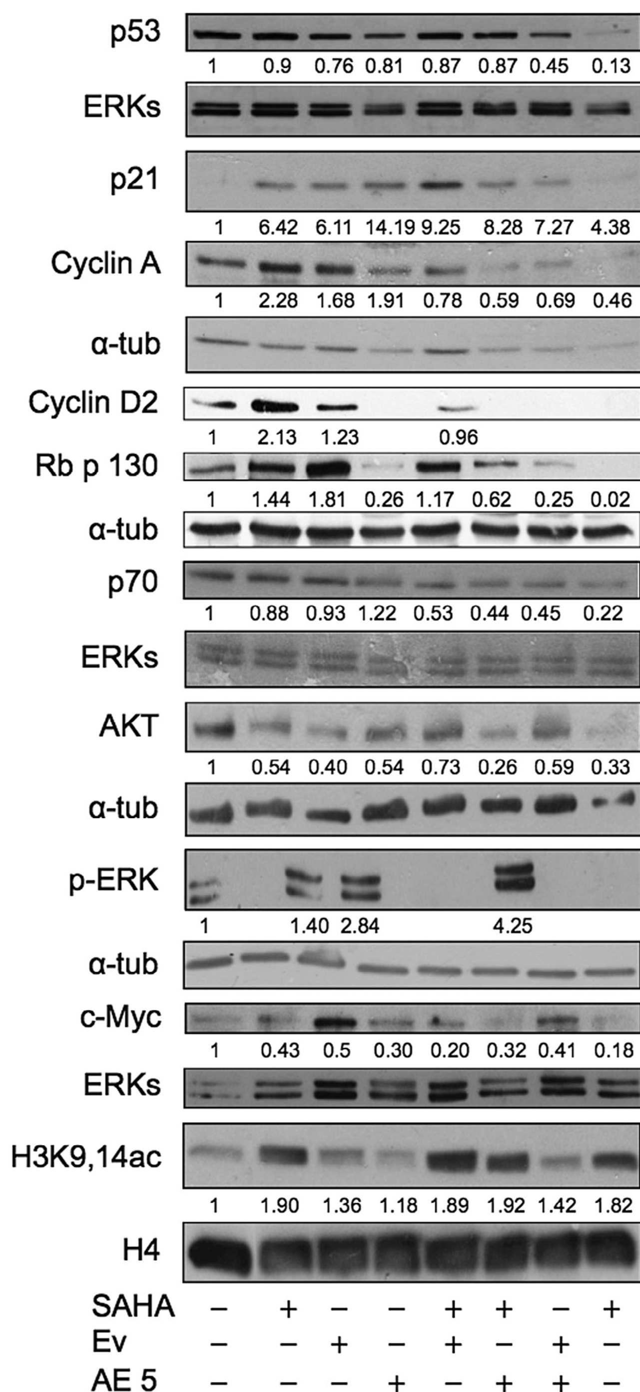

Fig. 5 – Molecular effects of anthocyanins in presence of Everolimus and SAHA in breast cancer cells. Western blot analysis of expression levels of the indicated proteins in MDA-MB-231 breast cancer cells after AE treatment at the indicated doses for 24 h. SAHA was used at 5 μM. Everolimus (Ev) was used as in Fig. 4. Total ERKs, α-Tubulin and histone H4 were used as loading control.

anticancer effects and (ii) chemo-preventive efficacy is maintained with a continuous anthocyanin intake. Further pharmacological studies should verify the efficacy and safety of higher concentrations.

5. Conclusions

The antineoplastic activity of AE was characterized in association with a genomic approach aimed at promoting the development of potato varieties with improved nutraceutical properties. Our insights into the effects of crude and AE extracts may also lead to a promising strategy for cancer recurrence prevention in patients in follow-up care after cancer treatment or with some forms of cancer predisposition. The Akt/mTOR signal transduction pathway negatively regulates granulocytic maturation, and it is likely that AE is able to reactivate differentiation in a time- and dose-dependent manner by quickly resetting the deregulated pathway in AMLs. Clearly, the molecular mechanism(s) by which mTOR signal transduction impacts on chromatin remodelling and modifications in these settings will need further experimental investigation. These findings are of interest in view of the impact of food consumption and functionalization on potential cancer treatment and prevention.

Conflict of interest

The authors declare no conflict of interest.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2015.09.063.

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